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14. ABSTRACT
The main conclusion from the research in the first year of funding is that PKC isozymes play a role in the control of the release of death factors from prostate cancer cells. It is clear that PKCs control the expression of mRNA for death factors in prostate cancer cells, and therefore there is great potential that PKC isozymes modulate either their transcription or mRNA stability. We succeeded in establishing the kinetics of CCL2 mRNA expression and release from LNCaP in prostate cancer cells in response to the phorbol ester PMA. We also found that CCL2 release occurs also in androgen-independent prostate cancer cells, and that this effect is mediated by PKCα and PKCδ isozymes. We also established a role for p38 MAPK in the release of CCL2 from LNCaP cells induced by PMA. Importantly, our research during the last year allowed to establish a novel paradigm which suggest that androgens regulate apoptotic factor release from prostate cancer cells in response to PKC activation.

15. SUBJECT TERMS
MCP-1 (CCL2), protein kinase C, androgens, death factors, apoptosis, prostate cancer cells

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INTRODUCTION

The main goal of our research supported by DOD is to elucidate the mechanisms by which protein kinase C (PKC) isozymes modulate apoptotic responses in prostate cancer cell models. The family of PKC isozymes comprises 10 different isozymes, which have been classified into 3 groups: "classical" or calcium dependent PKCs (cPKCs α, βI, βII, and γ), "novel" or calcium-independent (nPKCs δ, ε, η, and θ), and "atypical" (aPKCs ζ and λ/ι). Members of the first two groups can be activated by phorbol esters, natural compounds originally described as tumor promoters. cPKCs and nPKCs are the most prominent targets for the lipid second messenger diacylglycerol (DAG), a lipid generated mainly by phospholipase C isozymes upon activation of tyrosine kinase and G-protein-coupled receptors (GPCRs). Activation of these receptors leads to DAG generation which triggers the translocation and activation of cPKCs and nPKCs. PKC isozymes modulate important signaling pathways that control proliferation, differentiation, survival, apoptosis, and malignant transformation. While PKC has been initially viewed as growth-promoting, it became clear particularly in the last decade that these kinases could trigger growth inhibitory or apoptotic responses (1-3).

Most prostate cancer cell lines express cPKCα, nPKCδ and nPKCε. The roles of individual isozymes in mitogenesis and survival of prostate cancer cells are still a subject of debate. Our laboratory has determined that phorbol esters such as phorbol 12-myristate 13-acetate (PMA) cause apoptosis in androgen-dependent LNCaP prostate cancer cells via activation of PKCδ. PKCα may also be a player in the phorbol ester effect (4-6). Analysis of the mechanisms involved in phorbol ester-induced apoptosis in LNCaP cells pointed to the autocrine release of death factors as essential for triggering cell death. Indeed, conditioned medium (CM) collected from PMA-treated LNCaP cells (CM-PMA) induces a strong apoptotic response when added to a fresh culture of LNCaP cells, an effect not observed with CM collected from vehicle-treated cells (CM-Veh) (7). We have determined that the autocrine loop involves the secretion of two TNF family members, TNFα and TRAIL, and that interfering with TNFα and TRAIL receptors or their downstream effectors impairs the apoptotic effect of PMA. PKCδ has a dual role, both in the release of autocrine factors and as a downstream effector of death receptors (7). We have also determined that a combination of TNFα and TRAIL causes only a partial apoptotic response in LNCaP cells, suggesting the involvement of other factors. Using a commercial cytokine/chemokine array we found that PMA causes a prominent release of MCP-1 (Monocyte Chemoattractant Protein-1, also called CCL2) and IL-8 (interleukin-8). MCP-1/CCL2 appears to potentiate the apoptotic effects of TNFα and TRAIL. A main goal of our research is to understand the interplay between all these factors to induce an apoptotic response in prostate cancer cells.

Note: although in our grant application we used the name MCP-1, this cytokine will be referred as CCL2 in accordance with the current nomenclature.

BODY

1. PMA stimulates the secretion of CCL2 via activation of PKC: kinetics, quantitative analysis and effect of a PKC inhibitor. We have previously determined that several cytokines/chemokines were released from LNCaP cells in response to PMA. Indeed, an analysis of cytokines/chemokines CM-PMA compared to that of CM-Veh revealed marked differences (Fig. 1A). A limitation of the array approach is that it is not quantitative, thus it does not allow for a comparison of the relative amounts of factors released to the CM, as each antibody has its own sensitivity for detection. Nevertheless, an obvious increase in CCL2 and IL-8 was detected. Note that despite the 15-fold increase in TNFα levels in CM-PMA that we measured by ELISA (20 ± 1 pg/ml in CM-Veh *vs*. 297 ± 3 pg/ml in CM-PMA, Ref. 7), this is not clearly observed in the array (unless the blots are

overexposed, data not shown), most probably due to differential sensitivities of antibodies used by either method.

CCL2 has been initially characterized a as chemokine involved in the recruitment and activation of monocytes during acute inflammation, but work in recent years has also implicated CCL2 in apoptosis, survival, and the progression of several types of cancers (8, 9). To confirm that CCL2 is released to the CM in response to PMA, we measured CCL2 levels by ELISA (Fig. 1B). While basal levels of CCL2 in CM are under the detection limits (< 8 pg/ml), they were markedly elevated 8 h and 24 h after 100 nM PMA treatment (0.3 ng/ml and >30 ng/ml, respectively) (Fig. 1B). CCL2 secretion was blocked by pretreatment with the pan-PKC inhibitor GF109203X (bisindolilmaleimide I, 5 μM) (Fig. 1B); thus, the effect is PKC-dependent and not mediated by alternative phorbol ester receptors unrelated to PKC (1).

2. PMA stimulates the expression of CCL2 via activation of PKC: kinetics, quantitative analysis and effect of a PKC inhibitor. Q-PCR analysis revealed a significant induction of CCL2 mRNA levels 3 h after PMA treatment, and a robust induction (> 500-fold) at 6 h (data presented in our original application). We have now extended this finding to other prostate cancer cell lines, namely androgen-independent DU-145 and PC-3 cells (Fig. 1C). It seems that CCL2 mRNA levels are induced faster in these cell lines relative to LNCaP cells. Note that we have previously found that CM-PMA from DU-145 and PC-3 cells has apoptogenic activity when added to LNCaP cells (7). Thus, PMA is a strong inducer of CCL2 synthesis and release in prostate cancer cells.

3. Characterization of the role of individual PKC isozymes in CCL2 mRNA induction and release. To determine the role of specific PKC isozymes in CCL2 secretion in response to PMA (100 nM) we used pharmacological inhibitors and RNAi. Both the cPKC inhibitor Gö6976 (PKCα-selective in these cells, as PKCα is the only cPKC present in LNCaP cells) and the PKCδ inhibitor rottlerin dose-dependently inhibited CCL2 secretion induced by PMA (Fig. 2A and 2B). As non-specific effects have been reported for PKC inhibitors, we also use PKCα and PKCδ RNAi. We successfully depleted each of these PKC isozymes upon delivery of specific RNAi duplexes using Oligofectamine (Fig. 2C). We found that either PKC RNAi duplex was capable of inhibiting the release of CCL2 induced by PMA (Fig. 2D). We decided not to pursue the use of dominant-negative constructs, as originally proposed, since the results using RNAi are very reproducible, and RNAi is unquestionably more specific than dominant-negative PKCs to interfere with PKC function/expression in an isozyme-specific manner. Thus, both PKCα and PKCδ are involved in CCL2 secretion induced by PMA.

4. A p38 inhibitor blocks the release of CCL2 from LNCaP prostate cancer cells. We analyzed the role of p38 on the secretion of CCL2 from LNCaP cells. As shown in Fig. 3, the p38 inhibitor SB203580 caused a significant inhibition of CCL2 secretion. This result points to a role for p38 MAPK in CCL2 secretion in response to PKC activation.

5. CM-PMA not only activates p38 MAPK but also induces Akt dephosphorylation. It is well established that LNCaP cells have PTEN inactivated. This results in elevated Akt activity, which signals for survival. We established that PMA treatment causes a rapid dephosphorylation and inactivation of Akt in LNCaP cells. This effect is mediated by PKCα, and it occurs through the activation of a PP2A phosphatase (6). LNCaP cell death induced by PMA is markedly reduced when cells were engineered to express a constitutively activated Akt mutant (Myr-Akt). Therefore, it is plausible that Akt dephosphorylation is a prerequisite for the apoptotic effect of PMA. Fig. 4 shows that CM-PMA causes two prominent signaling effects: one is a significant activation of p38 MAPK, and the other is a fast dephosphorylation of Akt, as determined in either case by Western blot using phospho-specific antibodies. Thus, it is possible that the release of TNFα, TRAIL, and CCL2 causes

apoptosis by activation of the pro-apoptotic p38 MAPK pathway and by inhibition of the pro-survival Akt pathway.

6. The release of apoptotic factors from LNCaP cells is subject to androgen control. As LNCaP cells are androgen-dependent and we recently reported that androgens regulate the expression of PKCδ at a transcriptional level (10), we considered a very important issue to address whether androgen-depletion impairs the secretion of autocrine factors from LNCaP cells. We reasoned that in LNCaP cells growing in the absence of androgens the ability of phorbol esters to promote the autocrine secretion of death factors should be affected. To address this issue we collected CM-PMA from LNCaP cells growing either in normal medium or in steroid-depleted medium (charcoal-treated). We observed that, interestingly, the apoptogenic activity of CM-PMA collected from LNCaP cells growing in steroid-depleted medium was markedly reduced compared to that colleted from cells growing in normal medium. Addition of the synthetic androgen R1881 to the steroid-depleted medium restores the full apoptotic response of CM-PMA (Fig. 5). Therefore, it is conceivable that the secretion of apoptotic factors from LNCaP cells is regulated by androgens. Notably, the induction of TNFα mRNA by PMA was markedly reduced when LNCaP cells were grown in steroid-depleted medium. Addition of R1881 to the steroid-depleted medium restored the ability of PMA to induce TNFα mRNA levels (indeed levels were more than doubled compared to those observed in LNCaP cells growing in normal medium) (Fig. 6). While the effect of PMA on TRAIL mRNA induction was modest (~ 2-fold), it was also abolished in steroid-depleted conditions, and the addition of R1881 fully restored the PMA response (data not shown). Preliminary experiments revealed that the release of TNFα, TRAIL, and CCL2 from LNCaP cells, as determined by ELISA, was markedly reduced upon androgen-depletion. We wish to explore this issue in more detail.

KEY RESEARCH ACOMPLISHMENTS

- We established the kinetics of MCP-1 (CCL2) mRNA expression in LNCaP in prostate cancer cells in response to PMA.
- We established the kinetics of release of CCL2 to the CM in LNCaP cells in response to PMA using ELISA.
- We determined that CCL2 release in response to PMA occurs not only in LNCaP cells but also in androgen-independent PC3 and DU145 cells.
- Using a PKC inhibitor (GF109203X, or bisindoilmaleimide I), we determined that CCL2 secretion induced by PMA is mediated by PKC and not other phorbol ester receptor.
- Using isozyme-specific PKC inhibitors, we found that the PMA response (both CCL2 mRNA induction and release) is sensitive to PKCα and PKCδ inhibition.
- We successfully knocked down specific PKCs in LNCaP cells using RNAi.
- We determined using RNAi that both PKCα and PKCδ were involved in CCL2 secretion induced by PMA.
- We found that a p38 inhibitor blocks the release of CCL2 from LNCaP cells induced by PMA, suggesting a role for the p38 MAPK pathway in this response.
- We found that CM-PMA, when added to LNCaP cells, induces Akt dephosphorylation, suggesting that the released factors inhibit survival pathways.
- We established a new paradigm: the release of apoptotic factors in response to PKC activation is subject to androgen control. This finding may have significant implications.

REPORTABLE OUTCOMES

None.

CONCLUSIONS

The main conclusion from the research in the first year of funding is that PKC isozymes play a role in the control of the release of death factors from prostate cancer cells. It is clear that PKCs control the expression of mRNA for death factors in prostate cancer cells, and therefore there is great potential that PKC isozymes modulate either their transcription or mRNA stability. Although not specifically proposed in our original application, a highly related issue is how androgens control these effects, and preliminary data suggest that androgens play a major role in modulating the release of death factors in response to PKC activation. A main goal is to determine the functional implications of these findings. It is conceivable that CCL2 plays a role in modulating cell death, and that CCL2 and the pathways that control its release and effects could represent potential targets for prostate cancer therapeutics. We will continue with our studies to address the role of PKC isozymes and signaling pathways in the autocrine release of death factors as well as effectors, and we will pursue studies on apoptosis to determine the functional relevance of our findings.

We request approval to redirect a couple of aspects of the research proposed in our original application:

1. First, we believe that it would be important to carry out a microarray analysis of the genes controlled by PKC activation in LNCaP prostate cancer cells to assess their relevance in the paradigm studied here. We predict that this approach will simplify our identification of targets and potential effectors of CCL2, and data would certainly be highly informative and relevant to the current project. As we successfully depleted individual PKCs in LNCaP cells using RNAi, we are in a great position to identify isozyme-specific regulation of gene expression either by PKC activation, as well as by the release factors, including TNFα, TRAIL, and CCL2.

2. Also within the scope of the original application, we would like to pursue studies on how androgens modulate the pro-apoptotic autocrine loop induced by PKC activation. Our preliminary data revealing that the release of death factors in androgen-depleted LNCaP cells is impaired may have remarkable implications for the understanding of androgen-independence in prostate cancer, and therefore at this stage we are uniquely positioned to pursue mechanistic studies on this issue.

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Han, K.C., Lee, J., Marquez, V.E., and Kazanietz, M.G. Diacylglycerol (DAG)-lactones, a new class of protein kinase C (PKC) agonists, induce apoptosis in LNCaP prostate cancer cells by selective activation of PKCalpha. J. Biol. Chem. 277: 645-655 (2002).

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APPENDIX

6 figures and the corresponding figure legends.

FIGURE LEGENDS

Figure 1. Cytokine profile of vehicle and PMA-treated LNCaP cells. *Panel A*. LNCaP cells at approximately 75% confluence were treated with 100 nM PMA or vehicle for 1 h. CM-PMA and CM-Veh were collected 24 h later. Samples were tested for the presence of multiple cytokine and chemokines by a RayBio Human Cytokine Antibody Array 3.1 from RayBiotech, Inc. A marked increase in CCL2 (MCP-1) and IL-8 were observed in CM-PMA. *Panel B*. LNCaP cells were stimulated with PMA (100 nM, 1 h), and CCL2 levels in the CM-PMA were measured by ELISA at different times. When the PKC inhibitor GF109203X (5 μM) was used, it was added 30 min before and during PMA treatment. *Panel C*. LNCaP, DU145 or PC3 cells stimulated with PMA (100 nM, 1 h) or vehicle. CCL2 mRNA levels were analyzed by Q-PCR at different times. Data are expressed as fold-increase over basal levels and represents the mean ± S.E. of 3 independent experiments.

Figure 2. CCL2 release is mediated by PKCα and PKCδ. LNCaP cells were pre-incubated with either Gö6976 (*Panel A*), rottlerin (*Panel B*), or subject to PKCα or PKCδ RNAi (*Panels C* and *D*). Cells were treated with 100 nM PMA for 1 h, 30 min after the inhibitors were added or 48 h after transfection with the RNAi duplexes, and CM-PMA collected 24 h later. CCL2 levels in the CM were determined by ELISA. Results are the mean ± S.E. of 3 independent experiments. *Panel C* shows a representative Western blot for the depletion of PKC isozymes. *Control*, transfection with a control RNAi duplex.

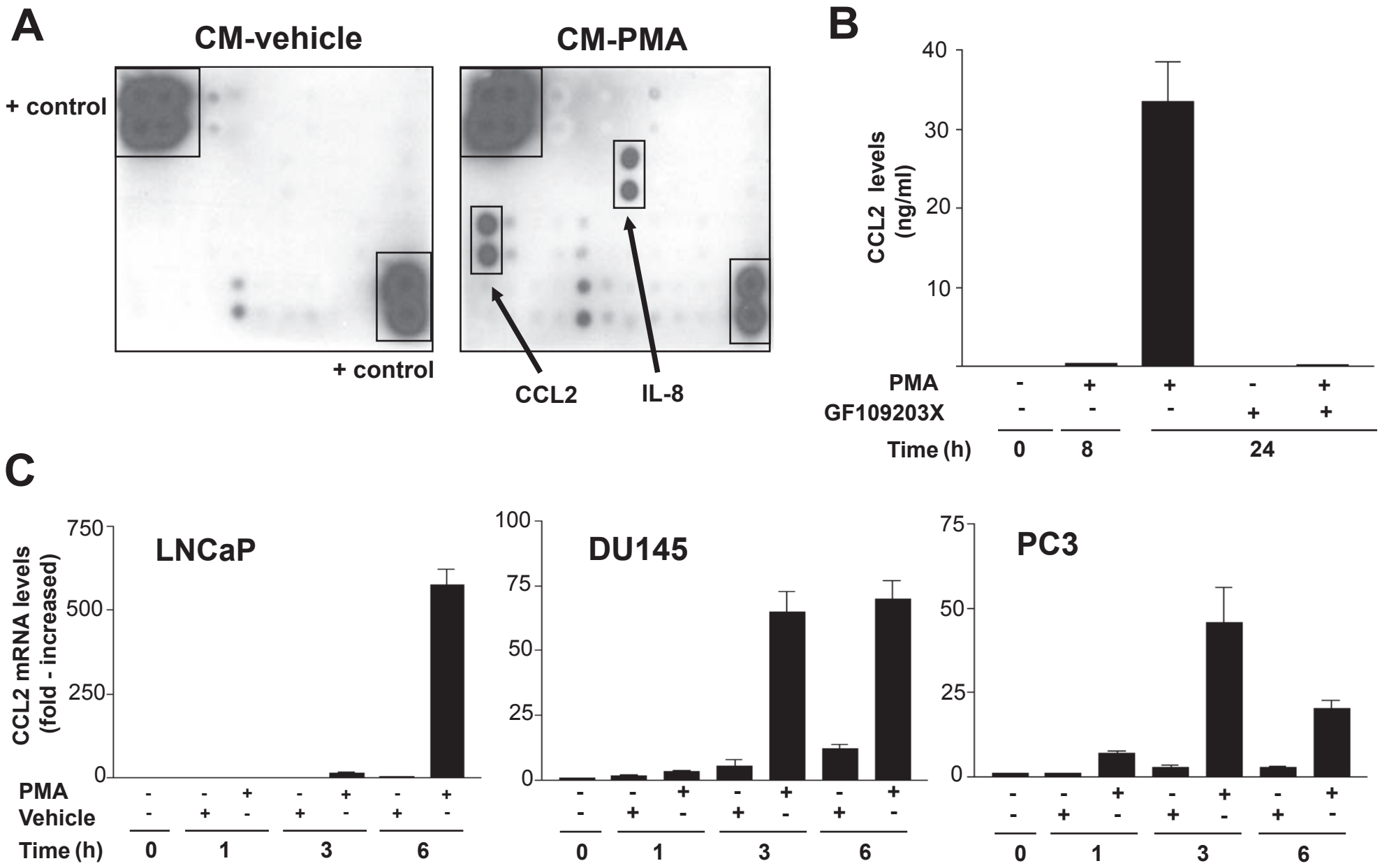
Figure 3. CCL2 release is mediated by p38 MAPK. LNCaP cells were pre-incubated with different concentrations of SB203580 and then treated with to 100 nM PMA for 1 h, and CM-PMA collected 24 h later. CCL2 levels in the CM-PMA were determined by ELISA. Results are the mean ± S.E. of 3 independent experiments.

Figure 4. CM-PMA activates p38 MAPK and inactivates Akt. LNCaP cells were treated with either CM-PMA or CM-Veh for the times indicated in the figure. Samples were analyzed by Western blot using anti-phospho- and anti-total- antibodies for p38 MAPK (*Panel A*) or Akt (*Panel B*).

Figure 5. Androgens modulate the activity of CM-PMA LNCaP cells were grown in normal, steroid-depleted medium (with charcoal-treated serum) or steroid-depleted medium plus R1881 (1 nM) for 48 h, and then treated with PMA (100 nM) or vehicle for 1 h. CM was collected 2 h later and added to a fresh culture of LNCaP cells. Twenty four h later cells were collected and subjected to DAPI staining. Results are presented as mean ± S.E (n = 3).

Figure 6. LNCaP cells were grown in normal, steroid-depleted medium (with charcoal-treated serum) or steroid-depleted medium plus R1881 (1 nM) for 48 h, and then treated with PMA (100 nM) or vehicle for 1 h. Cells were collected 3 h after PMA treatment and RNA was prepared using TRIzol (Invitrogen). TNFα mRNA levels were determined using real-time PCR with the TaqMan Gene Expression Assay (Applied Biosystems). The signal was normalized to the endogenous GAPDH. Results were expressed as fold-increase relative to untreated cells in normal medium.

Figure 1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+ control
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IL-8
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GF109203X
Time (h)
0
8
24
C
CCL2 mRNA levels (fold - increased)
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DU145
PC3
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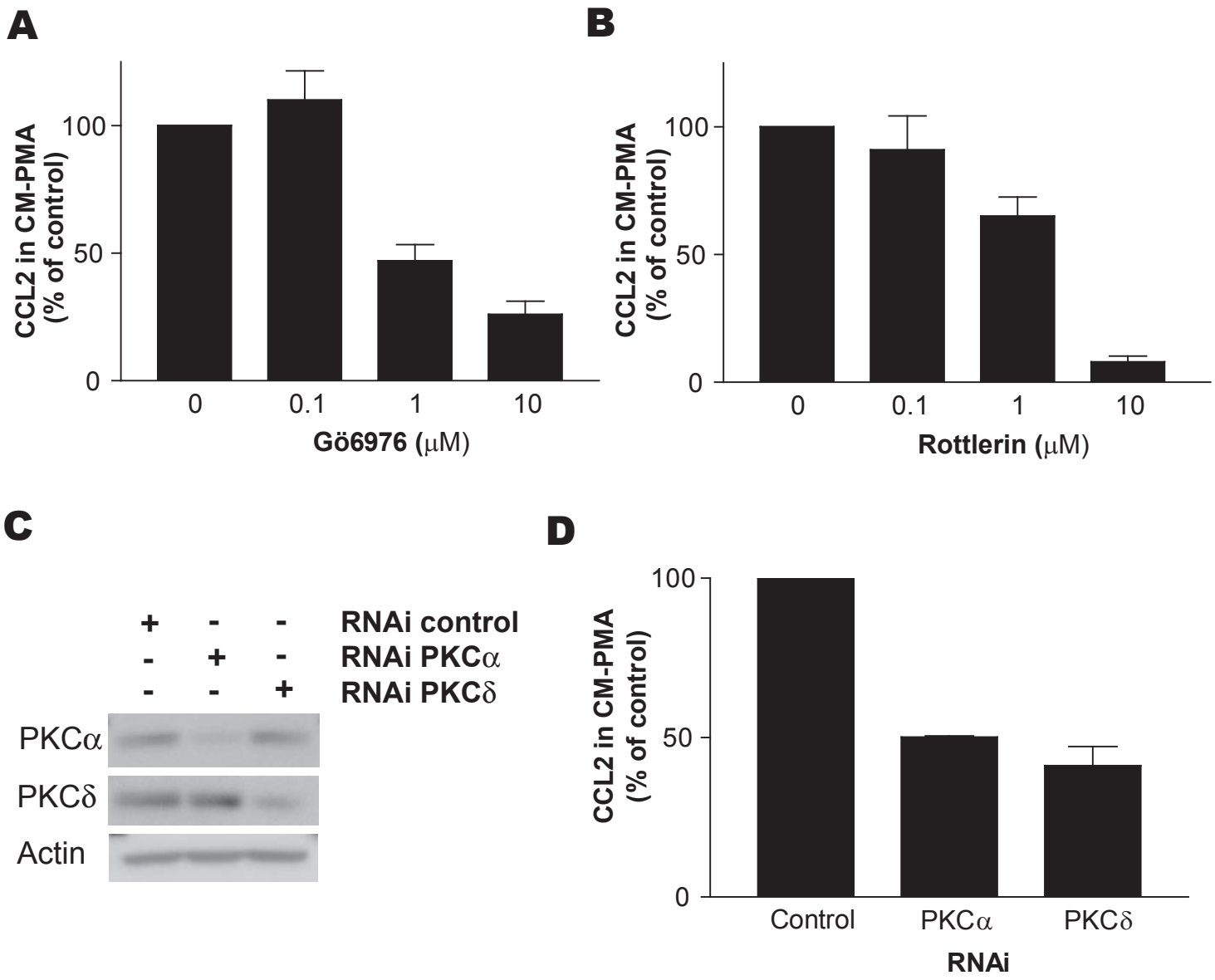
Figure 2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10
Gö6976 (μM)
B
CCL2 in CM-PMA (% of control)
100
50
0
0
0.1
1
10
Rottlerin (μM)
C
+ - - RNAi control
- + - RNAi PKCα
- - + RNAi PKCδ
PKCα
PKCδ
Actin
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50
0
Control
PKCα
PKCδ
RNAi

Figure 3

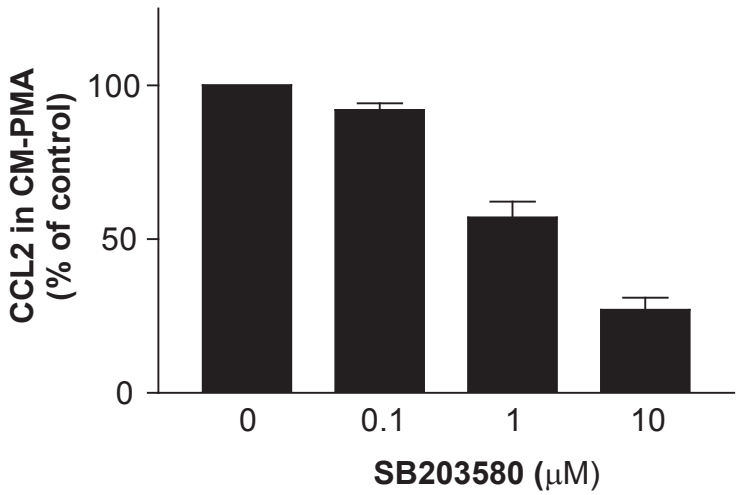
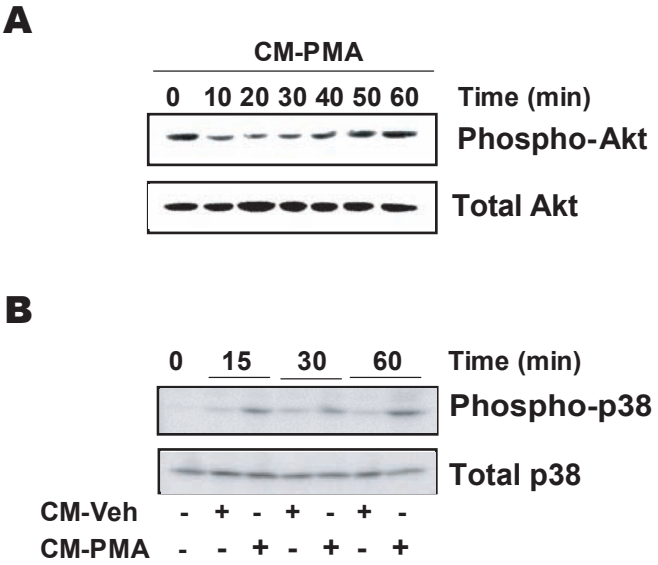


Figure 4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Phospho-Akt
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B
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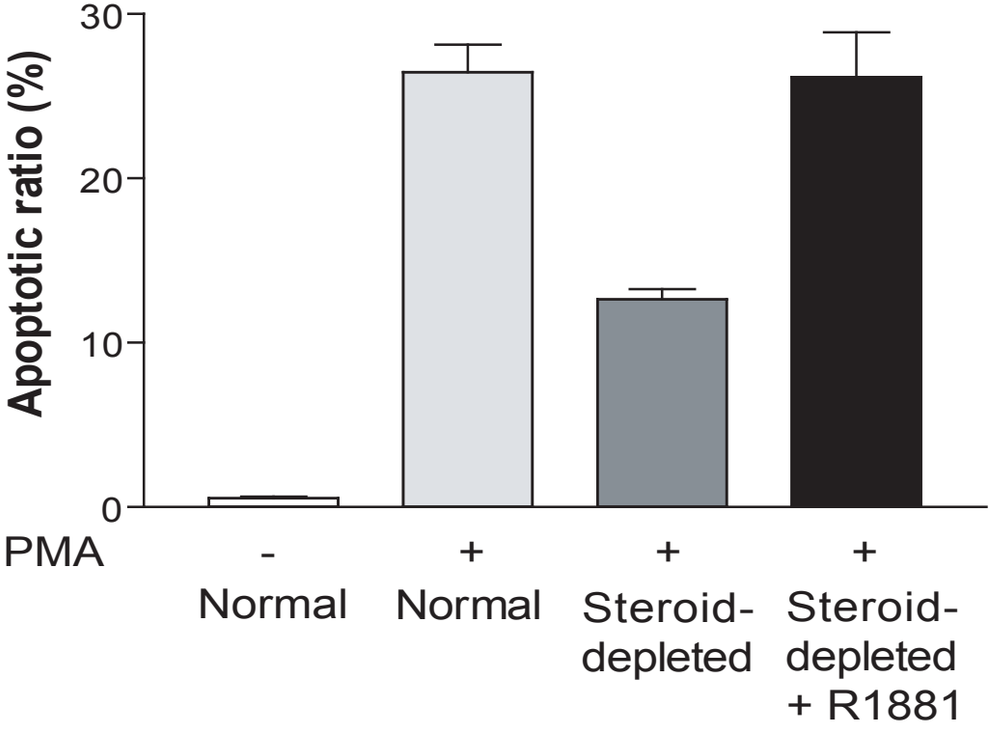
Figure 5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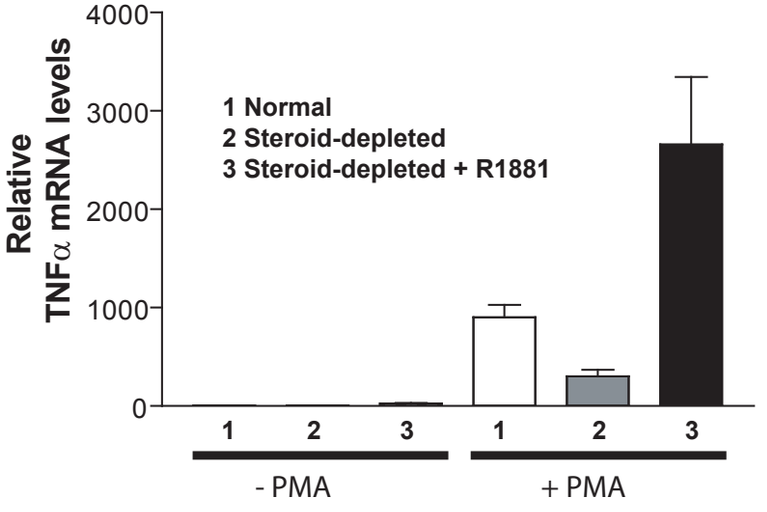
PMA
-
+
+
+
Normal
Normal
Steroid-depleted
Steroid-depleted + R1881

Figure 6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3000
2000
1000
0
1 Normal
2 Steroid-depleted
3 Steroid-depleted + R1881
1
2
3
1
2
3
- PMA
+ PMA